Light-Scattering Study of Effect of Electrolytes on α- and β-Casein Solutions

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Introduction

An attempt to determine, by the light-scattering method, the molecular weights of the main species, called α- and β-casein, into which whole casein has been separated in this laboratory (1, 2) revealed that their apparent molecular weights depend on the electrolyte concentration in the solution. The purpose of this communication is to show that this behavior of α- and β-casein, considered in conjunction with other properties (3), is evidence that they are not to be classed as "globular" or "native" proteins, in the sense in which these terms are understood when applied to unaltered ovalbumin, serum albumin, and the like, but resemble, instead, these same proteins in the denatured state. In the absence of added electrolyte a molecular-weight determination of casein is impracticable, for reasons that are discussed below, and in the presence of electrolyte casein solutions probably contain molecular aggregates. It is unlikely, therefore, that significant values for molecular weights will be found until a reliable means of dispersing these aggregates is developed.

Materials and Procedure

α- and β-Casein were prepared from unpasteurized cow's milk by the method of Warner (1) rather than by the shorter method of Hipp, Groves, Custer, and McMeekin (2), since there was the possibility that the latter, which employs differences in solubility in alcoholic or urea-containing solutions as the basis for separation, might convert an originally globular protein into a denatured final product. Warner's method depends on differences in the solubilities of the two components as functions of pH and temperature. The conditions are mild, and serious altera-

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tion of the starting material is considered unlikely. The only modifications made in the procedure were for the purpose of minimizing the chance of denaturation. Specifically, ether as a fat extractant was replaced by the less-soluble chloroform, and the final products were not dried, but were kept as pastes at 2°C., with toluene added as a preservative. Twelve reprecipitations were made to free the α-casein of all traces of the β-component. Electrophoretic analysis by J. H. Custer of this laboratory showed that both substances were free of detectable contamination.

β-Lactoglobulin was prepared from the whey by the method of Palmer (4). It was recrystallized three times by dialysis of sodium chloride solutions, and was stored as a paste at 2°C.

Ovalbumin was prepared from fresh eggs by the method of Sørensen and Høyrup (5). It was recrystallized four times from ammonium sulfate solution, dialyzed against distilled water at 2°, and dried from the frozen state.

Bovine serum albumin, Lot No. 128–125, was purchased from Armour and Company. It was stored at 2°C.

Light-scattering measurements were made by a method previously described (6). Solutions of ovalbumin, serum albumin, and lactoglobulin were clarified by filtration through an ultrafine sintered-glass filter, average pore diameter about 1 μ, directly into the light-scattering cell. The clarification of α-casein solutions presented some difficulties. The most highly clarified solutions were obtained by the following procedure. The casein was dissolved in dilute sodium hydroxide solution to a final pH of 7, then filtered successively through filter paper, a fine porosity sintered-glass filter, and an ultrafine sintered-glass filter. The solution at this point still contained particles, possibly a lipide–protein combination, which, on centrifuging, concentrated near the top of the centrifuge tube. This material was easily dislodged and it was difficult to withdraw the centrifuged solution without contamination by floating particles. Three extractions with chloroform removed the contaminant.[2] The solution was now centrifuged repeatedly for 1 hr. each time in a Spinco model L centrifuge at a speed of 40,000 r.p.m., corresponding to a maximum sedimenting force of approximately 140,000 $\times$ g. Nitrocellulose tubes were used, and the contents were removed by puncturing the tube about 1 cm. from the bottom with a hypodermic syringe and withdrawing slowly. After each centrifugation, the solution was filtered through an ultrafine filter and light-scattering was determined. A value was reached after three centrifugations which changed only a few per cent on centrifuging a fourth time. This clarified solution was used for the tests described below. At 436 mμ, the solution, whose concentration was 0.0134 g. of Na caseinate/ml., showed a value for Rayleigh's ratio[3] (corrected for solvent scattering) of 0.000621 cm.$^{-1}$. The repeated extraction and centrifugation caused a decrease of 19% in concentration. The latter was determined by evaporating an aliquot of solution to dryness on a steam bath, then drying to constant weight at 100°.

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[2] There is no evidence that this procedure materially affects the casein, since a preparation clarified by repeated centrifugation, without chloroform extraction, gave the same results.

[3] Rayleigh's ratio, $R = (I_{90}/I_0)(r^2/V)$, where I_{90} is the light intensity scattered at 90°, I_0 is the intensity of the incident beam, r is the distance of the observer from the source of scattered light, and V is the illuminated volume.

β-Casein was dissolved in dilute sodium hydroxide to pH 7. The solution was filtered through filter paper, then through an ultrafine sintered-glass filter. Centrifuging 1 hr. at 40,000 r.p.m. gave a solution whose light scattering was unchanged on centrifuging another hour at 40,000 r.p.m. The low-density material which caused trouble in the clarification of α-casein is not present in β-casein. At 436 mμ, the clarified solution, concentration 0.0172 g. Na caseinate/ml., showed a value for Rayleigh's ratio (corrected for solvent scattering) of 0.000488 cm.$^{-1}$. Concentration was determined as given above for α-casein. The high-speed centrifugations caused a decrease in concentration of about 2%.

Denatured ovalbumin, lactoglobulin, and serum albumin were prepared by heating 1% solutions, adjusted to pH 7 with ammonium hydroxide, for 3 min. in a boiling water bath. The denatured solutions were clarified by filtration through ultrafine sintered glass. Concentrations were determined as above.

The intensity of light of 436-mμ wavelength scattered by the clarified protein solutions, adjusted to pH 7 with dilute ammonia or acetic acid, was studied as a function of potassium chloride concentration over the range 0.02–0.1 M.[4] Time was allowed for the light scattering to reach an equilibrium value after adding potassium chloride.[5]

Results

Values of Rayleigh's ratio divided by protein concentration are plotted vs. electrolyte concentration in Fig. 1. The curves bring out the contrast between the caseins, on the one hand, (curves F, E), and native ovalbumin, serum albumin, and lactoglobulin, on the other (curves I, K). The scatterings of the native proteins are essentially independent of electrolyte concentration, while those of α- and β-casein increase with increasing concentration. Curve D shows that the aggregating effect is not specific to potassium chloride, since ammonium acetate also shows the effect, to a greater degree, in fact.[6] At pH 10 (curve H) the aggregating effect is less than at pH 7, but it is still present. Results at pH's 8

[4] Lower concentrations were avoided since, at very low electrolyte concentrations, it would be difficult to distinguish between aggregation and the effect of the electrolyte in diminishing the mutual repulsion of the charged protein particles. Both effects produce an increase in light scattering.

[5] Equilibrium was attained almost instantly for all but β-casein, denatured ovalbumin, and denatured serum albumin in 30% ethanol. For the first, an average period of about 45 min. was required. The other two required longer periods and were allowed to equilibrate 24 hr. For these three, a separate protein solution was used for each concentration of potassium chloride. Electrolyte-containing solutions of native ovalbumin and serum albumin, the latter both in water and 30% ethanol, showed no change in scattering in 24 hr.

[6] Bivalent cations caused precipitation at low concentrations. When magnesium chloride was added to a 0.4% α-casein solution, for example, precipitation began at a concentration of about 5×10^{-3} M.

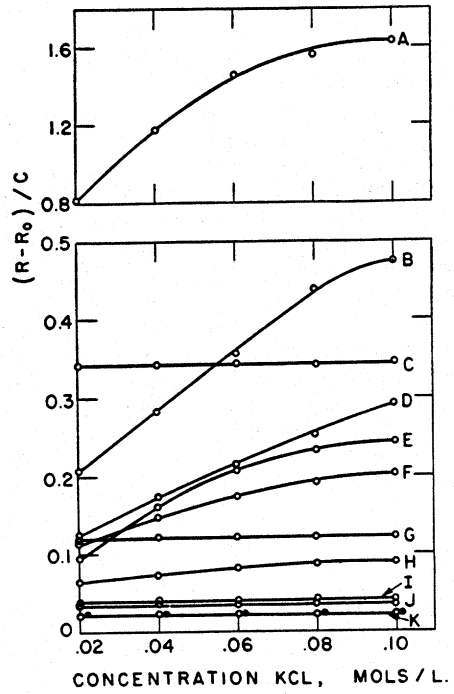

Fig. 1. Effect of potassium chloride concentration on light scattering of proteins. $(R - R_0)/c$, where $R - R_0$ is Rayleigh's ratio for the solution, corrected for solvent scattering, and c is the protein concentration, is plotted on the vertical axis.

A: denatured bovine serum albumin in 30% ethanol, c = 0.000584 g./ml.; B: denatured ovalbumin, c = 0.000763 g./ml.; C: denatured bovine serum albumin in water, c = 0.00112 g./ml.; D: α-casein, pH 7, c = 0.00668 g./ml., ammonium acetate used in place of potassium chloride; E: β-casein, c = 0.00291 g./ml.; F: α-casein, pH 7, c = 0.00668 g./ml.; G: denatured lactoglobulin, c = 0.00483 g./ml.; H: α-casein, pH 10, c = 0.00668 g./ml.; I: native bovine serum albumin in water, c = 0.00569 g./ml.; J: native bovine serum albumin in 30% ethanol, c = 0.00436 g./ml.; K: native ovalbumin (open circles), c = 0.00927 g./ml.; native lactoglobulin (filled circles), c = 0.01526 g./ml.

and 9 were intermediate between those at 7 and 10. At pH 3, on the acid side of the isoelectric point (lactic acid used for acidification), a similar effect was obtained. However, solutions at acid pH values were unstable, the light scattering increasing rapidly with time. The degree of aggregation was observed to depend on the concentration of protein. Thus, in curve F, where the concentration of α-casein is 0.00668 g./ml., the ratio

of the scattering in 0.1 M KCl to that in 0.02 M KCl is 1.78, whereas in another experiment, where the protein concentration was one-fourth of the above, the ratio was 1.49.[7]

Curve B shows that α- and β-casein, although contrasting with *native* ovalbumin, closely resemble *denatured* ovalbumin with regard to sensitivity to electrolytes. That aggregation by electrolytes is not a necessary property of denatured proteins, however, is proved by curves C and G, for denatured serum albumin and lactoglobulin, respectively. For these, the scattering is no more affected by electrolyte than for the corresponding native or globular forms. However, a contrast does exist between the native and denatured forms, and, in the case of serum albumin, this contrast can be brought out by adding alcohol before adding the electrolyte. The light scattering of native serum albumin in 30 % ethanol is unaffected by electrolyte concentration (curve J), while that of the denatured form in the same solvent (curve A) is markedly sensitive. In the terminology of colloid chemistry, the difference between the native and the denatured forms might be expressed by saying that the first is relatively lyophilic while the second is relatively lyophobic. For ovalbumin, the contrast between the forms is strong; for serum albumin, it is weaker, but can be brought out by adding a solvent which has less affinity for the protein than does water and also lowers the dielectric constant of the solution. Denatured lactoglobulin in 30 % ethanol is also sensitive to potassium chloride, but, unfortunately, it cannot conveniently be contrasted with native lactoglobulin under the same conditions, since native lactoglobulin denatures very rapidly in 30 % ethanol. Serum albumin, on the other hand, seems unaffected by 30 % ethanol, even after 24 hr. at room temperature.

α- and β-Casein are distinctly different from each other, as is illustrated by their amino acid compositions (7) and by a number of other properties. Two differences were observed in their aggregation by potassium chloride: one was in the time required to produce a constant light-scattering intensity after adding potassium chloride. For α-casein, this varied from

[7] The aggregating effect of electrolytes was greater for a highly clarified solution than for one less highly clarified. Thus, when the scattering, in the absence of electrolyte, of an α-casein solution was decreased fourfold by high-speed centrifugation, the aggregating effect of KCl, as judged by the ratio of the scattering of a solution containing 0.1 M to that of one containing 0.02 M, *increased* 17%. This shows that the aggregating action of KCl is on the casein itself, not on some impurity which can be removed by centrifugation.

less than 1 min. to about 5 min., while, for β-casein, it averaged about 45 min. Another difference was that the aggregation of α-casein was reversible, while that of β-casein was not. This is illustrated by an experiment in which the scattering of a casein solution containing 0.02 *M* KCl was measured. Then, a concentrated KCl solution was added so as to make 0.1 *M* KCl, with negligible change in volume, and light scattering was measured after equilibrium was reached. The KCl concentration was then returned to 0.02 *M* without change in protein concentration by dilution with protein solution; finally, the solution was made 0.1 *M* in KCl again. If the scatterings are normalized to a value of unity for the initial solution, the following sequences were obtained for α- and β-casein:

α-Casein

Concn. of KCl, moles/l.	0.02 → 0.1 → 0.02 → 0.1
Relative scattering	1.00 → 1.41 → 0.99 → 1.41

β-Casein

Concn. of KCl, moles/l.	0.02 → 0.1 → 0.02 → 0.1
Relative scattering	1.00 → 2.72 → 1.34 → 2.97

The α- and β-casein aggregates were not large enough to show appreciable dissymmetry of scattering. For example, a solution of α-casein containing approximately 0.004 g. of casein/ml. showed the following dissymmetry ratios ($I_{45°}/I_{135°}$) at 436 mμ: no KCl, 1.13; 0.01 *M* KCl, 1.07; 0.05 *M* KCl, 1.05; 0.1 *M* KCl, 1.04. β-Casein showed a corresponding effect. These results are easily understood if it is assumed that the clarified solution still retains small amounts of large-size impurities, which give rise to dissymmetry of scattering. If these impurities are unaffected by the addition of potassium chloride, then the aggregation of the casein brought about by the addition of electrolyte would diminish the relative contribution of the impurities, with the result that decreasing dissymmetry ratios would be observed.

Discussion

The effect of sodium chloride in increasing the light scattering of a casein solution was noted by Holwerda (8), who attributed it to a dehydrating effect, leading to an increase in refractive-index difference between solute and solvent. However, measurements of specific refractive increment (9) failed to reveal a measurable difference between solutions in 0.1 *M* potassium chloride and in water. The value found was

0.197 ml./g. at 436 mμ for the sodium salts of both α- and β-casein at pH 7. Moreover, such facts as the incomplete reversibility of the effect of potassium chloride on the light scattering of β-casein, the slowness of the scattering of β-casein solutions to reach equilibrium after adding potassium chloride, and the increasing effect of potassium chloride with increasing concentration of α-casein are difficult to explain on the refractive-index hypothesis, whereas these are familiar effects in aggregating systems.

The fact that α- and β-casein behave like denatured ovalbumin, serum albumin, and lactoglobulin (the latter two in solutions containing ethanol), but differ from the native forms of these proteins, suggests again that the caseins, in their natural state, are not proteins of the "globular" type, but resemble, rather, proteins in the denatured form. This supports the observation (3) that the optical rotation of casein, isolated from milk in the mildest possible fashion, is practically the same in 5 *M* guanidine hydrochloride solutions as in water, whereas the optical rotation of typically globular proteins is greatly increased by this reagent. The fact that the caseins are insoluble at the isoelectric point, both in water and in electrolyte solutions, is a further resemblance to denatured proteins, as is the fact that all attempts to crystallize them have failed. McMeekin (3) has also pointed out that casein resembles denatured or cooked proteins in ease of digestibility by proteolytic enzymes. Since the function of casein is infant nutrition, it is not surprising that it should occur in nature in easily digestible form.

The present study was undertaken primarily in connection with the determination of the molecular weights of α- and β-casein. The problem is complicated by the aggregation which electrolytes have been demonstrated to produce. Avoiding electrolytes would not necessarily solve the problem. Casein molecules cannot be studied in the uncharged state in water solution, owing to the insolubility of casein at its isoelectric point. Casein in aqueous solution is thus necessarily a polyelectrolyte, and the behavior of polyelectrolytes in the absence of added simple electrolytes exhibits complications which are due to the mutual repulsions of the unshielded charged centers—see, for example, the discussion by Fuoss (10) of the properties of synthetic polyelectrolytes with regard to osmotic pressure, light scattering, and viscosity. For this reason, determinations of the physical properties of proteins, at pH values removed from the isoelectric point, are almost always made in solutions containing added simple electrolyte.

Doty and Edsall (11) have discussed the light-scattering behavior of bovine serum albumin, in the absence of added electrolyte, at pH values where the protein bears a charge. They show that a straight-line plot for the usual light-scattering function Kc/R (where K is a constant involving, chiefly, the specific refractive increment, c is the protein concentration, and R is Rayleigh's ratio) vs. c is not to be expected, since the number of gegenions associated with the protein ion, and therefore the strength of the repulsive forces, will change with dilution. The experimental behavior at pH 3.2, where the protein ion carries a net charge of about 50 protons, shows this to be true. The initial slope of the plot is very steep, and falls off with increasing concentration. Thus, experimental points must be obtained at the highest possible dilutions in order for the extrapolation to arrive at a value corresponding to the correct molecular weight. It would be difficult to know whether one has gone to sufficiently great dilutions unless the correct value of the molecular weight were known in advance.

Several experiments were done by the present author on the light scattering of α-casein at pH 7 in the absence of added electrolyte. Results were erratic in that sometimes positive slopes were obtained, sometimes negative. The minimum protein concentration used in the measurements was 0.0003 g./ml. There was considerable scattering of the points, making it difficult to determine whether they formed straight lines or curves. Straight-line extrapolations led to molecular weights ranging from 25,000 to 65,000. In this connection, it should be noted that Doty and Steiner (12) found that the light scattering of serum albumin at pH 8 in the absence of electrolyte was not readily reproducible.

The difficulties discussed above apply to the value of 32,000 recently found for α-casein by the light-scattering method by D'yachenko and Vlodavets (13). The measurements were made at pH 9 (KOH), without added electrolyte. The most dilute solution contained 0.004 g. casein/ml., and a straight line was drawn through the points. Since it can be calculated from the data of Hipp, Groves, and McMeekin (14) that α-casein has a charge of about -37 per 32,000 molecular weight at pH 9, it would require considerable effort to establish the validity of any value found under the conditions mentioned.

The various values between 75,000 and 375,000 found for whole casein and certain fractions by means of the ultracentrifuge (15) were determined in phosphate buffer solutions and therefore probably represent aggregates.

Of perhaps greater validity is the value of 33,600 obtained for whole casein in 6.66 *M* urea by Burk and Greenberg by the osmotic-pressure method (16), since urea probably has a dispersing effect on the aggregates. However, much remains to be done before we have values for the caseins in which we can feel the same confidence that now applies to the values for serum albumin, lactoglobulin, and others of the crystallizable proteins.

Summary

Solutions of α- and β-casein are aggregated by electrolytes. This contrasts with the behavior of native ovalbumin, serum albumin, and lactoglobulin, but resembles that of the denatured forms of these proteins (the latter two after addition of ethanol). It is concluded that the caseins have the properties of denatured proteins. Determination of their molecular weights is complicated by the aggregation effect, and values in which confidence can be felt probably do not now exist.

References

1. Warner, R. C., *J. Am. Chem. Soc.* **66,** 1725 (1944).
2. Hipp, N. J., Groves, M. L., Custer, J. H., and McMeekin, T. L., *J. Dairy Sci.* **35,** 272 (1952).
3. McMeekin, T. L., *J. Milk and Food Technol.* **15,** 57 (1952).
4. Palmer, A. H., *J. Biol. Chem.* **104,** 359 (1934).
5. Sørensen, S. P. L., and Høyrup, M., *Compt. rend. trav. lab. Carlsberg* **12,** 12 (1917).
6. Brice, B. A., Halwer, M., and Speiser, R., *J. Opt. Soc. Amer.* **40,** 768 (1950).
7. Gordon, W. G., Semmett, W. F., Cable, R. S., and Morris, M., *J. Am. Chem. Soc.* **71,** 3293 (1949).
8. Holwerda, B. J., *Rec. trav. chim.* **50,** 601 (1931).
9. Brice, B. A., and Halwer, M., *J. Opt. Soc. Amer.* **41,** 1033 (1951).
10. Fuoss, R. M., *Discussions Faraday Soc. No.* **11,** 125 (1951).
11. Doty, P., and Edsall, J. T., *Advances in Protein Chem.* **6,** 35 (1951).
12. Doty, P., and Steiner, R. F., *J. Chem. Phys.* **20,** 85 (1952).
13. D'yachenko, P. F., and Vlodavets, I. N., *Kolloid Zhur.* **14,** 338 (1952).
14. Hipp, N. J., Groves, M. L., and McMeekin, T. L., *J. Am. Chem. Soc.* **74,** 4822 (1952).
15. Svedberg, T., Carpenter, L. M., and Carpenter, D. C., *J. Am. Chem. Soc.* **52,** 241, 701 (1930).
16. Burk, N. F., and Greenberg, D. M., *J. Biol. Chem.* **87,** 197 (1930).